Antiapoptotic Effect of Endothelin-1 in Rat Cardiomyocytes
In Vitro

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Abstract—Apoptosis of cardiac myocytes is thought to be a feature of many pathological disorders, including congestive heart failure (CHF) and ischemic heart disease (IHD). Because recent investigations indicate that endothelin-1 (ET-1) plays an important role in CHF and IHD, we investigated the effect of ET-1 on cardiomyocyte apoptosis. The presence of apoptosis in rat cardiomyocytes (H9c2 and neonatal) was evaluated by morphological criteria, electrophoresis of DNA fragments, 4′,6′-diamidine-2′-phenylindole staining, and TUNEL analysis. ET-1, but not angiotensin II, prevented apoptosis induced by serum deprivation via ET_A receptors in a dose-dependent manner (1 to 100 nmol/L). ET-1 also prevented cytochrome c release from mitochondria to the cytosol. The use of specific pharmacological inhibitors demonstrated that the antiapoptotic effect of ET-1 was mediated through a tyrosine kinase pathway (genistein and AG490) but not through protein kinase C (PKC; calphostin C), mitogen-activated protein kinases (PD98059 and SB203580), or PKA (KT5270) pathways. Adenovirus-mediated gene transfer of kinase-inactive (KI) c-Src reversed the antiapoptotic effect of ET-1. We further investigated whether Bcl-xL, an antiapoptotic molecule, would be upregulated by using a luciferase-based reporter system. ET-1 upregulated Bcl-xL, and this upregulation was inhibited by genistein or AG490 but not by calphostin C. The experiments with KI mutants for various tyrosine kinases revealed that c-Src and Pyk2 (but not JAK1, Jak2, Syk, and Tec) are involved in ET-1–induced upregulation of Bcl-xL expression. These findings suggest that ET-1 prevents apoptosis in cardiac myocytes through the ET_A receptor and the subsequent c-Src/Bcl-xL–dependent pathway. (Hypertension. 2003;41:1156-1163.)

Key Words: signal transduction — kinase — endothelin — apoptosis — myocardium

Cardiac myocyte cell death by apoptosis accompanies several heart diseases.1,2 It has been demonstrated in the myocardium from failing human hearts,3 in patients with dilated cardiomyopathy and arrhythmogenic right ventricular dysplasia,4,5 and in association with myocardial infarction.6 Apoptosis causes loss of contractile cells, compensatory hypertrophy of myocardial cells, and reparative fibrosis.7 Because a reduction of contractile material is a prominent feature in heart failure, modification of apoptosis in the myocardium might provide a new therapeutic target for cardiovascular diseases.

A number of stimuli induce a hypertrophic response in cardiac myocytes, including α-adrenergic agents, heparin-binding epidermal growth factor–like growth factor, insulin-like growth factor-1, leukemia inhibitory factor, neuregulin, cardiotoxin-1, angiotensin II (AII), and interleukin-1β.8–12 Several of these factors have also been shown to be proapoptotic, whereas others have an antiapoptotic role in cardiac myocytes.13,14

Endothelin-1 (ET-1), a family of 21–amino acid peptides, is 1 of the most potent hypertrophic stimuli for cardiac myocytes.15,16 Furthermore, a number of clinical and experimental investigations have demonstrated that ET-1 might play an important role in the pathophysiology of cardiovascular diseases, including congestive heart failure (CHF) and ischemic heart disease.17 The plasma and myocardial tissue levels of ET-1 increase in patients with CHF.18,19 In patients with acute myocardial infarction, plasma ET-1 levels are elevated20,21 and are correlated with 1-year mortality.21 We therefore hypothesized that ET-1 regulates apoptosis in the myocardium.

The effect of ET-1 on apoptosis is controversial. ET-1 has been reported to be an antiapoptotic factor in endothelial cells.22 On the other hand, there are studies of smooth muscle cells in which ET-1 causes apoptosis.23 In cardiac myocytes, ET-1 prevents oxidative stress– and β-adrenergic agonist–induced apoptosis.24,25 In the present study, we demonstrate that ET-1 prevents apoptosis induced by serum deprivation in cultured cardiac myocytes and investigate the signaling pathways that mediate the antiapoptotic effect of ET-1.
Methods

Cell Culture and Materials

The embryonic rat heart–derived myogenic cell line H9c2 was obtained from American Type Culture Collection (Rockville, Md). Rat neonatal cardiomyocytes were prepared from ventricles of 1-day-old Sprague-Dawley rats as described previously.26 The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin solution. The investigation was performed in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (Her Majesty’s Stationary Office, London, UK).

The cDNA for a kinase domain–deleted Tec was subcloned into an expression vector, pSRa.27 The kinase-deleted forms of porcine Syk (amino acids 1 to 504), human c-Src (amino acids 1 to 253), and mouse Jak1 (amino acids 1 to 879) were amplified by the polymerase chain reaction and inserted individually into the same vector. The expression plasmid for the kinase-deleted Jak2 was constructed as described previously.27 Expression plasmids for a kinase-inactive (KI) form of Pyk2 were described previously.31 Adenoviruses containing either the β-galactosidase cDNA (Ad.LacZ) or a cDNA encoding chicken KI-c-Src was prepared, amplified, and purified as described previously.27 Human ET-1 was purchased from the Peptide Institute Inc. Antibodies against Bcl-xL, Bcl-2, c-Src (clone GD11, ED10), and an activated form of c-Src ([pY418] phosphospecific antibody) were purchased from Santa Cruz Inc, Upstate Biotechnology Inc, and Biosource International, respectively. Antibodies against phosphosignal transducer and activator of transcription 3 (Stat3 [Tyr705]) and Stat3 were purchased from Cell Signaling Technology Inc. BQ123, BQ788, genistein, AG490, calphostin C, KT5270, PD98059, SB203580, and PP2 were purchased from Calbiochem. The remaining reagents including AII were obtained from Sigma unless otherwise indicated.

DNA Laddering

To evaluate DNA fragmentation, cellular fragmented DNA was extracted by the Triton X-100 lysis method, which efficiently eliminates intact chromatin. Floating and/or adherent cells were collected, and DNA fragments were extracted, fractionated by 1.8% agarose gel electrophoresis, and stained with ethidium bromide.30

DAPI Staining

Cells were fixed in 3% paraformaldehyde in phosphate-buffered saline for 20 minutes and stained with a solution of 4′,6-diamidino-2-phenylindole (DAPI; 10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 100 mmol/L NaCl, 500 ng/mL DAPI) for 10 minutes at room temperature. The apoptotic cells were evaluated under a fluorescent microscope.31

TUNEL Analysis

Cells were fixed and then labeled using terminal deoxyribonucleotidyl transferase according to the manufacturer’s instructions (in situ apoptosis detection kit, Wako).

Detection of Cytochrome C Release

Cell lysates were prepared for the detection of cytochrome c in cytosolic and mitochondrial fractions, and detection of cytochrome c release was performed by Western blot analysis with an anti-cytochrome c antibody according to the manufacturer’s instructions (cytochrome c releasing apoptosis assay kit, Biovision).

Western Blot Analysis

Expression levels of Bcl-xL, Bcl-2, c-Src, the activated form of c-Src, Stat3, and the phosphorylated form of Stat3 were analyzed by Western blot analysis. In brief, cells were lysed in a modified radioimmunoprecipitation assay buffer (10 mmol/L HEPES, pH 7.4, 5 mmol/L EDTA, 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 100 μmol/L NaVO₄, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, and fresh 0.5 mmol/L PMSF and 10 μg/mL leupeptin). Cell lysates were prepared by scraping, sonication, and centrifugation for 20 minutes at 14 000 rpm in a microfuge at 4°C. Cell lysates were subjected to 5% to 20% SDS-polyacrylamide gradient gel electrophoresis. The separated proteins were electrophoretically transferred onto nitrocellulose membranes, and the resultant blots were incubated with the first antibody for 2 hours, followed by incubation for 1 to 2 hours with the secondary antibody (horseradish peroxidase conjugated). Immunoreactive bands were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech UK Ltd).

Transfection and Luciferase Assay

Transfections were performed with the Tfx-50 lipofectin reagent (Promega). In brief, cells were plated at 10⁶ cells/well in DMEM supplemented with 10% fetal calf serum (FCS) in 6-well plates and allowed to attach overnight. Transfections were performed 1 day after seeding by using a combination of 1.5 μg of expression plasmids, 1.5 μg Bcl-xL-luc, 0.3 μg pRL-TK (Promega), and 9.9 μL Tfx-50. Cells were deprived of serum for 16 hours and then treated with 100 nmol/L ET-1 for 5 hours. Cell lysates were prepared, and the activity of Photinus pyralis luciferase was measured with the dual-luciferase reporter assay system (Promega) and normalized by the activity of Runilla reniformis luciferase.

Statistical Analysis

Data are expressed as the mean±SD. For comparisons between multiple groups, we determined the significance of differences between group means by ANOVA with the least significant difference for multiple comparisons. P<0.05 was considered statistically significant.

Results

Effect of ET-1 on Serum Deprivation–Induced Apoptosis

We first examined the effects of ET-1 on apoptosis in cultured cardiomyocytes. Electrophoresis of DNA fragments showed that 100 nmol/L ET-1 prevented formation of the characteristic apoptosis ladder induced by serum deprivation in both H9c2 cells and rat neonatal cardiomyocytes (Figure 1A). In contrast, AII, which is a vasconstrictive peptide similar to ET-1, had no effect on apoptosis in H9c2 cells, although these cells express the angiotensin II type 1 (AT₁) receptor.32 The antiapoptotic effect of ET-1 was dose dependent over the range used (1 to 100 nmol/L; Figure 1B). We confirmed that ET-1 prevented serum deprivation–induced apoptosis by TUNEL analysis and DNA-binding dye (DAPI) staining (Figures 1C through 1E). Serum deprivation reduced cell viability, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (data not shown). Furthermore, ET-1 prevented serum deprivation–induced cytochrome c release from mitochondria to the cytosol (Figure 2).

The effects of ET-1 are initiated by their binding to G protein–coupled heptahelical receptors, ETₐ and ETₐ, expressed in a wide variety of tissues and cells.33 To identify which ET receptor (ETₐ or ETₐ) is responsible for the antiapoptotic effect of ET-1 in H9c2 cells, we used selective ETₐ and ETₐ receptor antagonists. The antiapoptotic effect of ET-1 was almost completely inhibited by treatment with the selective ETₐ receptor antagonist BQ123, but not by the ETₐ receptor antagonist BQ788 (Figures 3A and 3B). These observations indicate that ET-1 participates in the survival of cardiac myocytes by preventing apoptosis by way of the ETₐ receptor in myocytes.
Effects of Various Signaling Inhibitors on ET-1–Prevented Apoptosis

Because the intracellular protein kinases mediate the prevention of apoptosis in cardiac myocytes,1,2,3,4 we next examined whether protein kinases were involved in the antiapoptotic effect of ET-1 in H9c2 cells by using tyrosine kinase inhibitors (genistein and AG490), protein kinase C inhibitors (calphostin C), mitogen-activated protein (MAP) kinase inhibitors (PD98059 for extracellular-regulated kinase and SB20380 for p38-MAP kinase), and a cAMP-dependent kinase inhibitor (KT5270). Genistein and AG490 inhibited the effect of ET-1, whereas calphostin C, PD98059, SB20380, and KT5270 failed to show any effect (Figures 4A and 4B). In addition, treatment with the phosphatidylinositol-3 kinase inhibitor wortmannin showed no effect on ET-1–prevented apoptosis (data not shown). These results suggest that ET-1 prevents serum deprivation–induced apoptosis in cardiac myocytes through a tyrosine kinase–dependent mechanism.

c-Src Involved in the Antiapoptotic Effect of ET-1

Recent evidence suggests that the c-Src family of protein tyrosine kinases is involved in apoptotic cell death in certain types of cells.35,36,37 We therefore hypothesized that c-Src might participate in the antiapoptotic effect of ET-1. To determine whether c-Src activity was regulated by ET-1, cells were stimulated by 100 nmol/L ET-1 for varying amounts of time, and c-Src activity was analyzed by Western blotting by using an antibody that selectively recognizes the activated form of c-Src. The activity of c-Src clearly increased by 4.5-fold within 30 seconds in response to ET-1 stimulation and then declined (Figure 5, top). We confirmed that there were no significant changes in c-Src protein levels of the same amount of cell lysates (Figure 5, bottom).

To further investigate the role of c-Src in the antiapoptotic effect of ET-1, adenoviruses were used to overexpress either β-galactosidase (Ad.LacZ) or a KI–c-Src. Transfection of H9c2 cells with a KI–c-Src, but not with Ad.LacZ, increased c-Src in...
a concentration-dependent manner (Figure 6A). Expression of KI–c-Src significantly inhibited the antiapoptotic effect of ET-1, whereas expression of β-galactosidase with Ad.LacZ had no effect (Figures 6B and 6C). These results indicate that c-Src is required for the antiapoptotic effect of ET-1.

**Effects of KI Mutants for Various Tyrosine Kinases on Bcl-xL Expression**

Because c-Src has been shown to negatively regulate apoptosis via Bcl-xL, an antiapoptotic molecule, in several cell types,35–37 we investigated whether Bcl-xL was involved by using a luciferase-based reporter system. Western blot analysis showed that ET-1 stimulated Bcl-xL expression (Figures 7A and 7B). ET-1 upregulated Bcl-xL gene expression, which was inhibited by treatment with genistein or AG490, but not by calphostin C (Figure 7C). The experiments with KI mutants for various tyrosine kinases showed that KI–c-Src completely inhibited ET-1–induced Bcl-xL gene expression (Figure 7D). In addition, KI-Pyk2 partially inhibited its expression, whereas KI-JAK1, KI-Jak2, KI-Syk, or KI-Tec showed no effect. These findings suggest that c-Src and Pyk2 are involved in Bcl-xL expression induced by ET-1.

**ET-1 Stimulates STAT3 Phosphorylation**

Because it has been reported that STAT3 regulates Bcl-xL expression in cardiac myocytes,38 finally we examined whether ET-1 stimulates STAT3 phosphorylation. ET-1 clearly stimulated STAT3 phosphorylation in a time-dependent manner, and this STAT3 phosphorylation was inhibited by treatment with a specific c-Src inhibitor, PP2 (Figure 8).
The major findings of this study are that ET-1 prevents apoptosis induced by serum deprivation in a dose-dependent manner via an ETA receptor in H9c2 cardiomyocytes and that the antiapoptotic effect of ET-1 is mediated through a c-Src/Bcl-xL pathway. Evidence for this proposal includes the following: (1) ET-1, but not AII, prevented mitochondrial cytochrome \( c \) release and apoptosis induced by serum deprivation in a dose-dependent manner, and this antiapoptotic effect was inhibited by an ETA receptor antagonist (BQ123) but not by an ETB receptor antagonist (BQ788); (2) the inhibitory effects of ET-1 on apoptosis were inhibited by tyrosine kinase inhibitors and adenovirus-mediated overexpression of KI\(-c\)-Src; (3) ET-1 stimulated c-Src activation; and (4) ET-1 upregulated an antiapoptotic molecule, Bcl-xL, and this upregulation was inhibited by tyrosine kinase inhibitors or cotransfection with KI\(-c\)-Src.

Recent evidence suggests that apoptosis of cardiac myocytes is a feature in cardiovascular diseases, including CHF and myocardial infarction. The levels of plasma and myocardial ET-1 increase in patients with CHF and myocardial infarction, suggesting the critical role of ET-1 in these cardiovascular disease states. Therefore, we investigated whether ET-1 affects myocardial apoptosis in this study. We showed here that ET-1 prevents serum deprivation-induced mitochondrial cytochrome \( c \) release and apoptosis, suggesting that the antiapoptotic effect of ET-1 is mediated through a mitochondrial apoptotic pathway. We further demonstrated that ET-1 prevents apoptosis in a dose-dependent manner via the ETA receptor. The effects of ET were mediated through 2 distinct receptor subtypes of \( G \) protein–coupled receptors, termed ET\(_A\) and ET\(_B\), expressed in a wide variety of cells and tissues. In myocardium, ET\(_A\) receptors are mainly expressed, and small amounts of ET\(_B\) receptors are expressed. Consistent with the expression levels in the myocardium, ET\(_A\) receptors act as a major pathway for several effects of ET-1, such as myocardial contraction and hypertrophy. Similar to these effects of ET-1, our findings indicate that the antiapoptotic effect of ET-1 in cardiac myocytes is also mediated via the ET\(_A\) receptor.

A number of proapoptotic and antiapoptotic signaling pathways in cardiac myocytes have been demonstrated. To investigate the molecular mechanisms of the antiapoptotic effect of ET-1 in cardiac myocytes, we used an adenovirus-based vector system that allows for highly efficient DNA transfection in many cell types. The efficiency of expression examined with Ad.LacZ or Ad.KI-c-Src for 1 hour at 37°C, incubated with DMEM supplemented with 10% FCS for 48 hours. Cell lysates were prepared and analyzed by Western blotting with anti-c-Src antibody. We demonstrated that overexpression of KI\(-c\)-Src reversed the antiapoptotic effect of ET-1, suggesting that c-Src plays a critical role in the ET-1–mediated antiapoptotic pathway in cardiac myocytes.

**Figure 5.** ET-1 stimulates c-Src activation. After H9c2 cells were deprived of serum in the presence or absence of 100 nmol/L ET-1 for the indicated periods, cell lysates were prepared. Anti-phosphospecific-c-Src (top, active c-Src) and c-Src antibody (bottom, c-Src) were used to quantify these proteins by Western blotting of whole lysates that were obtained in the same experiments. Relative c-Src kinase activity was quantified by densitometry. Results are representative of 3 independent experiments.

**Figure 6.** Overexpression of Ad.KI-c-Src inhibits antiapoptotic effect of ET-1. A, H9c2 cells were infected with either Ad.LacZ (10 and 100 m.o.i.) or Ad.KI-c-Src (1, 10 and 100 m.o.i.) for 1 hour at 37°C and then incubated with DMEM supplemented with 10% FCS for 48 hours. Cell lysates were prepared and analyzed by Western blotting with anti-c-Src antibody. B, Cells were infected with 100 m.o.i. of either Ad.LacZ or Ad.KI-c-Src for 1 hour at 37°C, incubated with DMEM supplemented with 10% FCS for 48 hours, and then deprived of serum (FCS) in the presence of 100 nmol/L ET-1 for 24 hours. Fragmented DNA was extracted from cells as described in the legend to Figure 1. M indicates DNA size markers. Results are representative of 3 independent experiments. C, Bar graphs show means±SD of 1200 to 1600 cells of 3 independent experiments (DAPI staining). *P<0.01 vs control.
Bcl-xL plays a critical role in the antiapoptotic signaling pathway in a variety of cells, including cardiac myocytes. In addition, recent investigations have suggested that c-Src regulates Bcl-xL in several cell types. Therefore, we next focused on Bcl-xL expression in the antiapoptotic pathway by ET-1. We demonstrated that ET-1 upregulated Bcl-xL expression, and this upregulation was completely inhibited by both KI-c-Src and tyrosine kinase inhibitors, which inhibited the antiapoptotic effect of ET-1. These findings suggest that c-Src is an upstream molecule for Bcl-xL expression in cardiac myocytes. c-Src itself has also induced the activation of several signaling molecules, including MAP kinase and STAT3, and both can stimulate Bcl-xL expression. Regarding this, Araki et al recently reported that ET-1 prevents apoptosis induced by \(/H9252\)-adrenergic agonists, and this effect is inhibited by treatment with the MAP kinase inhibitor PD98059. This difference might be due to apoptosis-inducing stimuli, because the \(/H9252\)-adrenergic agonist itself stimulates MAP kinase activity in cardiac myocytes. Downstream from c-Src, STAT3 is another molecule that might stimulate Bcl-xL expression. Karni et al \(^{37}\) reported that c-Src positively regulates Bcl-xL expression in cardiac myocytes. c-Src itself has also induced the activation of several signaling molecules, including MAP kinase and STAT3, and both can stimulate Bcl-xL expression.\(^{36,38}\) This difference might be due to apoptosis-inducing stimuli, because the \(/H9252\)-adrenergic agonist itself stimulates MAP kinase activity in cardiac myocytes.\(^{44}\)

Figure 7. Effects of KI mutants for various tyrosine kinases on Bcl-xL expression. A, H9c2 cells were deprived of serum for 24 hours in the presence or absence of ET-1. Cell lysates were prepared, and expression of Bcl-xL and Bcl-2 was analyzed by Western blotting, as described in Methods. B, Relative Bcl-xL expression was quantified by densitometry. Results are representative of 2 independent experiments. C, After pBcl-xL/luc (1.5 \(\mu\)g) and pRL-TK (0.3 \(\mu\)g) were introduced into cells, they were deprived of serum for 16 hours and pretreated with 0.1% dimethyl sulfoxide (cont), 10 \(\mu\)mol/L genistein (Gen), 100 \(\mu\)mol/L AG490 (AG), or 1 \(\mu\)mol/L calphostin C (CalC) for 1 hour; then cells were treated with 100 nmol/L ET-1 for 5 hours. Cell extracts were subjected to luciferase assay as described in Methods. The activity of Photinus pyralis luciferase was normalized by activity of Runilla reniformis luciferase. Bar graphs show mean±SD \((n=4)\). *\(P<0.01 vs\) control. D, After pBcl-xL/luc (1.5 \(\mu\)g) and pRL-TK (0.3 \(\mu\)g) were introduced into cells together with 1.5 \(\mu\)g each of blank vector (Vector), expression plasmid for control vector (vector), or the KI form of each protein tyrosine kinases (Src, Jak1, Jak2, Tec, Pyk2, Syk), cells were deprived of serum (FCS) for 16 hours, and then treated with 100 nmol/L ET-1 for 5 hours. Cell extracts were analyzed as described above. Bar graphs show mean±SD \((n=6)\). *\(P<0.01 vs\) control.

Figure 8. ET-1 stimulates STAT3 phosphorylation. A, After H9c2 cells were deprived of serum (FCS) in the presence or absence of 100 nmol/L ET-1 for indicated periods, cell lysates were prepared. B, After cells were pretreated with 10 \(\mu\)mol/L PP2 for 1 hour, they were stimulated with ET-1, and then cell lysates were prepared. Anti-phospho-STAT3 and STAT3 antibody (STAT3) were used to quantify these proteins by Western blotting. Results are representative of 2 independent experiments.
cardiomyocyte apoptosis is unknown. Further investigation is required to understand the precise mechanisms of the antiapoptotic signaling pathway by ET-1 in the myocardium.

In summary, we demonstrated a novel signaling pathway for the antiapoptotic effect of ET-1 in cardiac myocytes. ET-1 prevents serum deprivation–induced apoptosis in cardiac myocytes via the ET\(_4\) receptor. c-Src is activated by ET-1, upregulates Bcl-x\(_I\) expression, and shows an antiapoptotic effect in cardiac myocytes. Collectively, these findings indicate a potentially important role for the c-Src/Bcl-x\(_I\) pathway in the antiapoptotic effect of ET-1. Because the loss of contractile cardiac myocytes due to apoptosis results in a further decrease of cardiac function, identification of the signaling pathway that mediates survival and/or apoptosis in cardiac myocytes is important. Thus, our data provide new insight into the molecular basis and therapeutic target for further decrease of cardiac function, identification of the signaling pathway that mediates survival and/or apoptosis in cardiac myocytes.

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